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Analysis of the protein products encoded by variant glucokinase transcripts via expression in bacteria

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Five variant transcripts of the single rat glucokinase gene have been described that are naturally expressed in islets of Langerhans, liver and anterior pituitary. Four of these were prepared as cDNA and expressed in bacteria in order to begin to address their physiological roles. Expression of constructs pGKB1 (normal islet/pituitary glucokinase) and pGKL1 (normal liver glucokinase) resulted in a glucose-dependent, glucokinase-like activity, 7-fold and 45-fold, respectively, above background. Expression of pGKB3 (variant islet/pituitary glucokinase) and pGKL2 (variant liver glucokinase) in contrast, did not result in any glucokinase-like activity.

Glucokinase; Islets of Langerhans; Liver; Anterior pituitary; Bacterial expression

1. INTRODUCTION

The glucose phosphorylating enzyme glucokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1) plays a central role in regulating glucose metabolism in liver and pancreatic islets of Langerhans [1,2]. The single glucokinase gene in the rat genome can be transcribed and processed to yield several distinct species of mRNA [3–5]. The major glucokinase mRNA in liver has a size of 2.4 kb, as opposed to the major species in islets, which is 2.8 kb. Analysis of cDNA [4–8] and genomic [4,8,9] clones has revealed that the mature mRNAs are derived from 10 exons, and while both the liver and islet transcripts contain exons 2 through 10, exon 1 is unique and is spliced in a tissue-specific manner. Recently, 3 additional variant glucokinase transcripts have been described that are expressed in islets and islet cell lines [7], anterior pituitary cells and cell lines [7], solid insulinoma tumors [4], and liver [8]. The goal of the work described in this manuscript was to develop a system for producing large quantities of protein from both normal and variant glucokinase transcripts in order to begin to evaluate their physiological significance.

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2. MATERIALS AND METHODS

2.1. *Cloning of glucokinase cDNA isoforms into the bacterial expression vector pTrc99A*

The bacterial vector system chosen for these studies is pTrc99A, a derivative of pKK.322, as described by Amann et al. [10]. This vector utilizes a Trp/Lac promoter to drive expression of the heterologous gene of interest. The plasmid contains the lacIQ repressor, which allows expression of the heterologous gene to be tightly controlled by the presence or absence of IPTG.

Insertion of the normal islet glucokinase cDNA [5,7] into the pTrc99A vector was facilitated by site-directed mutagenesis [11], introducing an *Nco* I site at the ATG start codon (the first of two in-frame AUG triplets encountered in the open reading frame of islet glucokinase [4,5,7]) and a *Bam* HI site in the 3′ untranslated region, 41 bp 3′ of the TGA stop codon. Introduction of the *Nco* site (mutation of the sequence AGATGC to CCATGG) results in a base change in the second codon leading to the conservative substitution of a valine for a leucine in the translated product. This construct was designated pGKB1.

To generate the liver specific glucokinase cDNA, PCR amplification was carried out on liver poly (A^+) RNA as previously described [7], using a 5′ oligonucleotide, 5′ACGGGATCCTTTGCAACACTCAG C3′ from the unique 5′ untranslated region of the liver glucokinase cDNA [6] and 3′ oligonucleotide 5′ACGGAATTCCCCTTGGTCC AATTGAG3′ that encompasses nucleotides 980–996 [7]; included in the oligonucleotide sequences are restriction sites (*Bam* HI and *Eco* RI) that facilitated cloning of the PCR products. An *Nco* I site was introduced at the ATG start codon of this DNA, but without alteration of the second codon (mutation of AGATGG to CCATGG). This plasmid was designated pGKL1.

The variant glucokinase cDNA transcript expressed in islets and anterior pituitary cells that has the unique 5′ untranslated region and N-terminal sequence associated with islet glucokinase cDNAs, but that also contains a 52-nucleotide deletion corresponding to the 3′ end of exon 2 [7] was subcloned by a strategy similar to that employed for construction of pGKB1. This plasmid was designated pGKB3.

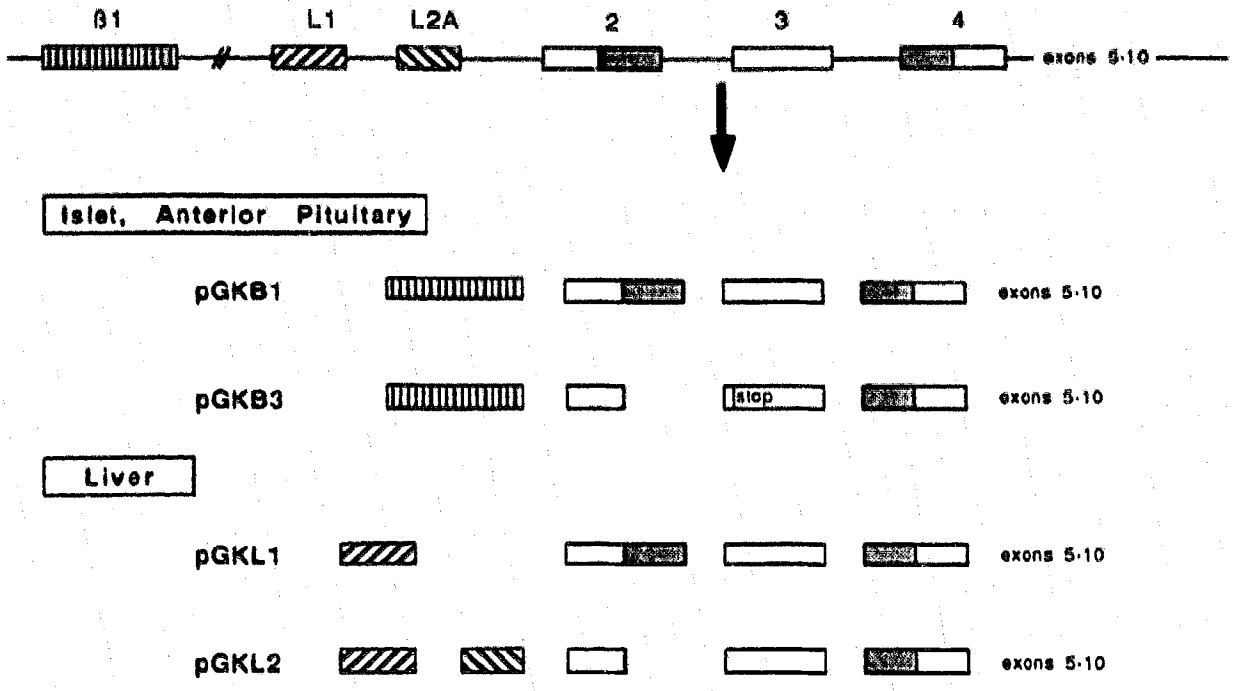


Fig. 1. Schematic summary of cDNA constructs expressed in bacteria. The top bar depicts the structure of the rat glucokinase gene [4,8,9]. The gene is processed to yield distinct mRNA species containing segments of the gene as indicated, in islet and anterior pituitary cells (the cDNA versions of these were cloned into bacterial vector pTre99A to yield constructs pGKB1 and pGKB3) and liver (constructs pGKL1 and pGKL2).

The variant glucokinase cDNA clone from rat liver [8] that contains a 151-bp insertion between exons 1 and 2 of the normal transcript, as well as the same 52-bp deletion as found in the islet/anterior pituitary construct pGKB3 was subcloned by introduction of the *Nco* I site as described for pGKL1, and the resultant plasmid was designated pGKL2.

The glucokinase cDNA variants used in this study are summarized schematically in Fig. 1.

2.2. *Expression of the GK isoforms in bacteria and preparation of extracts*

Competent *E. coli*, strain JM109 were transformed with the constructs described above and plated on LB/ampicillin to select for transformants. For expression studies, 2-ml cultures were grown for 6 h in LB/ampicillin and diluted 1:40 into duplicate sterile tubes containing 10 ml LB/ampicillin. Cultures were allowed to grow to log phase (0.3 OD at 600 nM) at which time IPTG (final concentration of 1 mM) was added to one of the duplicate cultures (induced) while the other received no additions (uninduced). Both cultures were then grown overnight at 37°C with shaking.

The overnight cultures were centrifuged at 8000 × *g* and the pellets brought up in 1/10 volume of GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) with 3 mg/ml lysozyme, left at room temperature for 5 min, transferred to ice, and supplemented with PMSF (0.2 mM), Nonidet-40 (0.1%), DTT (1 mM), and NaCl (0.25 M). The samples were then immediately sonicated and cleared by a 10 min spin in a microcentrifuge at 4°C. The protein concentration was measured using Bradford's reagent (Bio-Rad).

2.3. *Preparation of region-specific antipeptide antibodies and Western blot analysis*

Two synthetic polypeptides were prepared (courtesy of Sarah Stadley and Dr. Lilah Gierasch, University of Texas Southwestern Medical Center) one (GK-1, LDDRARMEATKKEK) with sequence corresponding to the N-terminal 15 amino acids of the islet glucokinase isoform [4,5,7], and the other (GK-2, KLHPSFKERFHASVR) corresponding to amino acids 414–428 encoded by constructs pGKL1, pGKL2 and pGKB1, but not pGKB3. The peptides were coupled to PPD (purified protein derivative of tuberculin) with glutaraldehyde and used to raise anti-peptide antibodies in White New Zealand rabbits as previously described [7]. The polyclonal antiserum against GK-1 (specific for the islet isoform of glucokinase) is designated U343 and that against GK-2 (common region) is termed V980. For Western blot analysis, the polyclonal sera were affinity-purified, first by passage over a Staphylococcus protein A column, followed by passage over a column containing the peptide to which the sera were raised, using protocols and reagents supplied by Pierce. Electrophoresis of bacterial extracts, transfer to nitrocellulose and immunoblotting were carried out as described [7], except that washing of blots after addition of the secondary antibody was done with 5% instead of 2% Tween-20.

2.4. *Glucokinase enzymatic assays*

Assays were carried out at 37°C, in a reaction buffer consisting of 0.1 M Tris, pH 7.2, 5 mM ATP, 10 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 500 μM NAD and glucose, in a range of concentrations from 0.5 to 50 mM. The assay buffer also contained 2 μl (2.5 U) of glucose-6-phosphate dehydrogenase, prepared from *Leuconostoc mesenteroides* (Boehringer Mannheim) [12]. The reaction was initiated by addition of 20 μl of the cleared supernatant from the crude bacterial extract and change in absorbance at 340 nm was monitored over 10 min. Addition of bacterial extracts to the reaction buffer containing NADH revealed no degradative effect of the extract on NADH.

3. RESULTS

3.1. *Expression of glucokinase variants in E. coli*

Bacterial expression of the 4 glucokinase constructs shown in Fig. 1 was evaluated by Western blot analysis.

As shown in Fig. 2(left), antibody U343, raised against the islet/pituitary glucokinase-specific peptide GK-1 detected a protein of approximately 52000 Da as the product of plasmid pGKB1, but did not detect an IPTG-inducible protein from the other 3 constructs. Antibody V980 (raised against the common region peptide GK-2) detected proteins of approximately 52000 Da expressed from both plasmids pGKL1 and pGKB1 as shown in Fig. 2(right). In addition, V980 detected a larger product with apparent molecular mass of 56000 Da expressed from the variant glucokinase construct pGKL2, in keeping with its predicted size of 54773 Da [8]. In efforts to optimize detection of the small (8000 Da) peptide predicted by construct pGKB3, analysis was carried out by the following techniques: (1) gradient gel electrophoresis (10–20% acrylamide) and blot transfer, a technique designed for resolution of smaller peptides; (2) gradient gel electrophoresis, followed by

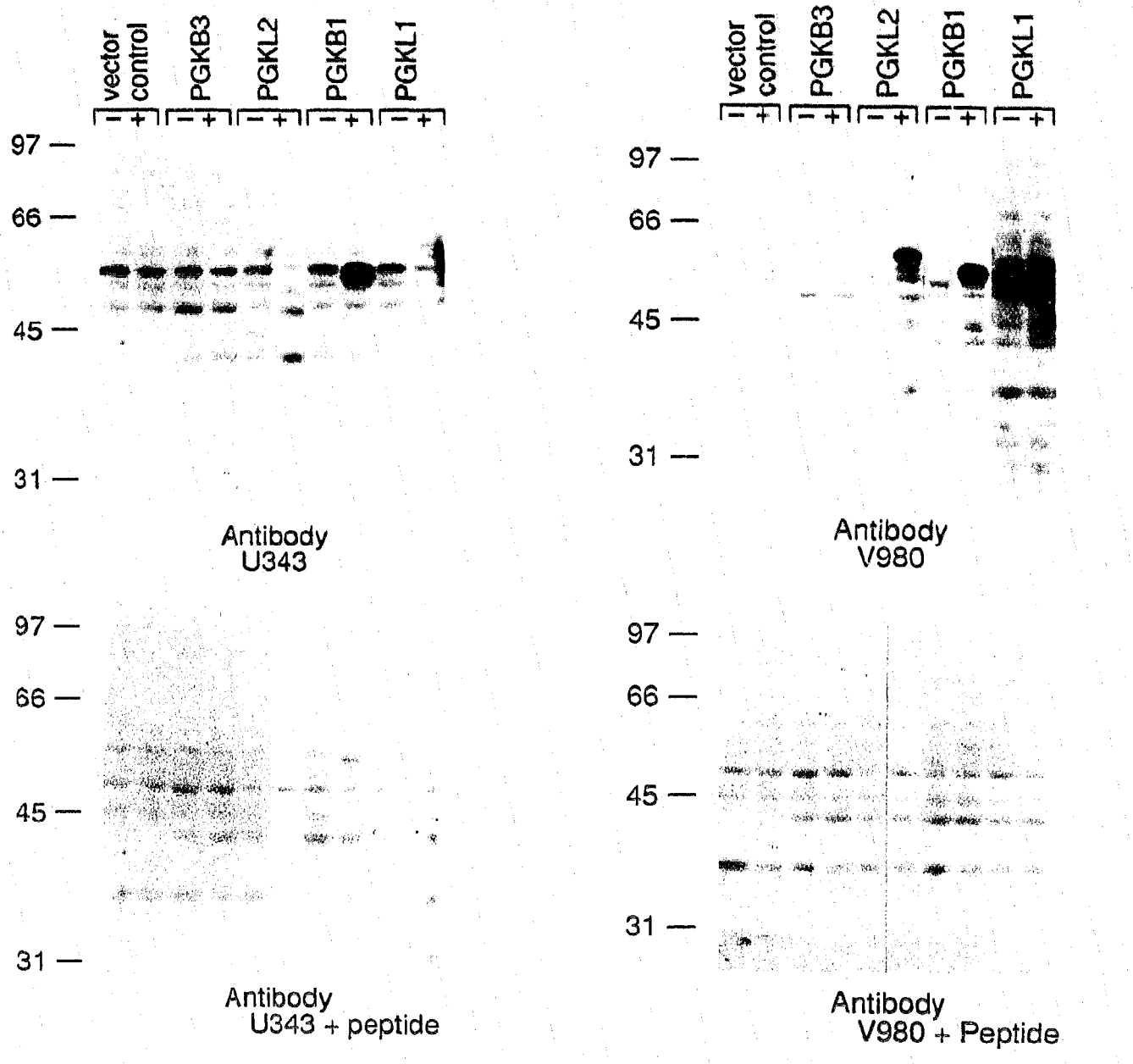
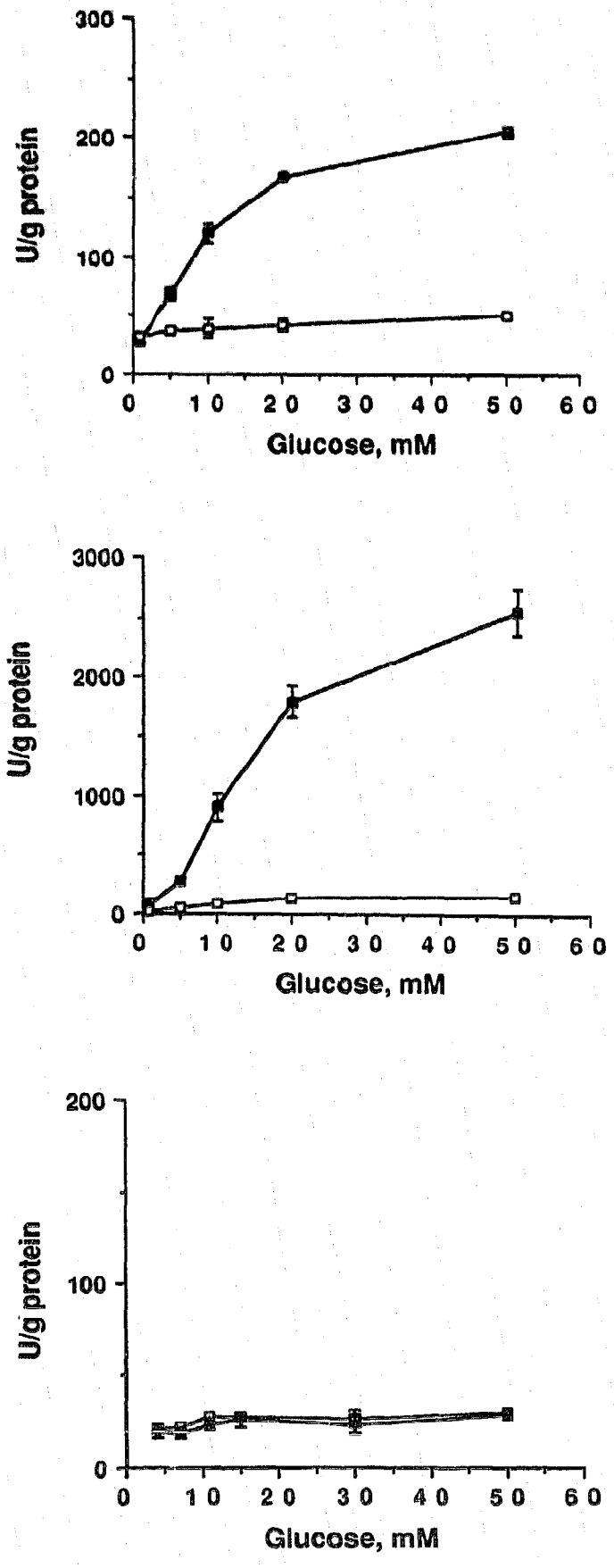


Fig. 2. Western blot analysis of glucokinase variants expressed in bacteria. Left: bacteria containing the variant constructs were grown in the presence (+) or absence (−) of IPTG. Western blot analysis was carried out as described in Section 2 with immunopurified U343 (top panel) or with U343 after preincubation with peptide Gk-1 (lower panel). Right: Western blot analysis was carried out as in panel A, except with immunopurified V980 (top panel) or with V980 after preincubation with peptide GK-2 bottom panel). Positions of molecular mass standards (values in kDa) are indicated on the left of the figures.



glutaraldehyde fixing of the gel prior to blot transfer, in an effort to transfer the peptide as a large, cross-linked complex; and (3) metabolic labeling and immunoprecipitation, carried out both with the standard and specialized electrophoresis techniques described above. Despite this extensive analysis, we were unable to detect expression of the peptide predicted by pGKB3, a result in keeping with its apparent absence from eucaryotic cells in which its mRNA is found [7]. It should be noted that the constructs were verified by sequencing prior to expression studies. Also, expression of each of the 4 constructs in bacteria gave rise to detectable RNA transcripts (data not shown).

3.2. *Catalytic activity of glucokinase isoforms*

Fig. 3 shows activity measurements performed on bacterial extracts expressing the variant glucokinase cDNAs. Extracts of bacterial cultures grown in the absence of the transcriptional inducer IPTG exhibit a low rate of glucose phosphorylation that is independent of assay glucose concentration over the range 1–50 mM. This low background activity, which is likely due to bacterial hexokinase, is also present in cultures containing the pTrc99A plasmid without a cDNA insert (data not shown). In the presence of IPTG, a glucose-dependent glucokinase activity is clearly seen that reaches a maximum of 200 U/g protein for plasmid pGKB1 (7.5-fold above background; Fig. 3, top) and 2564 U/g protein for plasmid pGKL1 (45-fold above background; Fig. 3, middle) at 50 mM glucose. Densitometric scanning of the blot in Fig. 2(right) reveals that the pGKL1 (liver) protein is present at 6.5-fold greater abundance than the pGKB1 (islet) protein when assayed with the common antibody V980 indicating that the 10-fold difference in activity is largely a function of the amount of glucokinase protein expressed.

Measurement of glucose phosphorylating activity was also performed on extracts of bacteria containing plasmids pGKL2 and pGKB3. For construct pGKL2, glucose phosphorylating activity was found to be at the low background level in the presence or absence of IPTG (Fig. 3, bottom) in spite of the fact that the level of expression of the pGKL2 protein product was similar to pGKB1. Not surprisingly, no high K_m glucose phosphorylating activity was detected in bacteria transformed with pGKB3 (data not shown).

Fig. 3. Glucose phosphorylating activity in bacterial cultures expressing variant glucokinase cDNAs. For all panels, crude extracts were prepared from bacteria grown in the presence (■) or absence (□) of IPTG and assayed for glucose phosphorylating activity as described in section 2. Top: activity in extracts prepared from bacteria expressing plasmid pGKB1. Middle: activity in extracts prepared from bacteria expressing plasmid pGKL1. Bottom: activity in extracts prepared from bacteria expressing plasmid pGKL2. Data are expressed as the mean activities ± SEM in 3 independent bacterial extracts.

4. DISCUSSION

Glucokinase has been ascribed a central role in the regulation of hepatic and islet β-cell glucose metabolism. Recently, work from several laboratories [3-9] has resulted in the identification of 5 distinct variant glucokinase mRNA species expressed in islets, liver and anterior pituitary. In the current study, we have begun to address the physiological significance of the multiple glucokinase gene products by preparing four of these naturally occurring transcripts as cDNA and expressing them in bacteria. Absent from this group is a glucokinase cDNA described by Magnuson and Shelton [4] cloned from a solid insulinoma tumor library that contains a 51-bp deletion at the 5′ end of exon 4, that would be designated clone pGKB2 using recently suggested nomenclature [13]. This clone was not included in the present study because extensive analysis of islet, anterior pituitary, AtT-20 and insulinoma cell line RNA in our laboratory by polymerase chain reaction amplification and nucleotide sequencing failed to identify any clones containing this deletion [7].

Expression of constructs pGKL1 and pGKB1 resulted in a glucose-dependent, glucokinase-like activity 7.5-fold and 45-fold, respectively above the background bacterial glucose phosphorylation activity when assayed at 50 mM glucose. The greatly enhanced liver glucokinase activity is in keeping with the more efficient expression of the liver isoform protein as measured by Western blot analysis. One possible explanation for the enhanced expression of the liver isoform is codon usage, since the codon GTG (Valine), which is found at the second position of the islet glucokinase constructs pGKB1 and pGKB3 is rarely found in bacterial genes, and results in approximately 3-fold less expression when inserted into test genes compared to the GCT (Alanine) codon [14], found at position 2 in the liver glucokinase constructs pGKL1 and pGKL2. Other factors may also be at work, as suggested by comparison of the two liver constructs pGKL1 and pGKL2. While the latter is expressed at a level approximately equal to islet glucokinase, it is clearly less efficiently expressed than pGKL1, despite the fact that the two constructs share the GCT second codon. This discrepancy might be explained by the fact that the 151-bp insertion in pGKL2 contains two AGA codons for arginine [8] whose corresponding tRNAs are extremely rare; genes containing this codon at their 5′ end are expressed with reduced efficiency [15].

The pGKL2 gene product does not encode an enzymatically competent glucokinase protein. In this naturally occuring mRNA, a 151 bp insertion introduces a total of 87 unique amino acid residues relative to pGKL1. Of these, 50 residues are encoded by the insert itself and 37 residues come from nucleotides derived from a region of exon 2 that is shared with pGKL1, but that is read in a different frame as a consequence of the insertion [8]. Interestingly, although pGKL2 contains a 52 nucleotide deletion corresponding to the 3′ end of exon 2, the putative binding sites for the substrates ATP and glucose [6] are left untouched, suggesting that important functional domains that determine activity are intact. It should be noted, however, that the 87 altered amino acids are found just 8 residues to the N-terminal side of the proposed ATP binding site; furthermore, the inserted sequence is highly hydrophobic compared to the sequence of the active glucokinase isoforms. Although the exact structural perturbations introduced by the insertion are currently unknown, it appears that these alterations are sufficient to cause the variant protein to be nonfunctional as a glucose phosphorylating enzyme. While we present no proof of expression of the 8000 Da peptide predicted by the alternate transcript of islets and anterior pituitary, pGKB3, we would not predict that it can encode an active enzyme, since the open reading frame in this transcript terminates upstream of the region containing the putative ATP and glucose binding sites.

The physiological role of the variant products of the glucokinase gene remains an open question, since although the transcripts are naturally occurring, they do not appear to result in efficient production of protein in mammalian cells. Immunoblotting with a polyclonal antibody raised against the purified liver glucokinase protein detects only a single band of identical molecular weight in liver and islets [3,16]. Further, extensive efforts aimed at demonstrating the expression of the 58 amino acid peptide predicted by construct pGKB3 in islet or anterior pituitary cells and cell lines have thus far been unrewarding [7]. It should be noted, however, that levels of the variant glucokinase mRNA in liver are regulated by fasting and refeeding [8], suggesting that there may be physiological conditions that lead to accumulation of one or both of the variant proteins. Finally we considered the possibility that the variant constructs might encode proteins capable of regulating glucose phosphorylation, but found that mixing of either variant with pGKB1 or pGKL1 or with crude extracts of liver or islets gave strictly additive effects (data not shown).

While glucokinase is not classically thought of as an allosterically regulated enzyme (in contrast to hexokinase, which is potently inhibited by glucose-6-phosphate), evidence for regulation of glucokinase activity has recently begun to accumulate. Van Schaftingen and co-workers have described a fructose phosphate-sensitive glucokinase regulatory protein [17]. Interestingly, this regulatory system appears to be less active in islets than in liver [18]. Other ligands, such as long-chain acyl CoAs [19] or alloxan [20], or enzymes of covalent modification, such as protein kinase A [21] have been implicated as potential regulators of glucokinase activity, but have never been assayed in a comparative study of the liver and islet enzymes, in part

because of the difficulties inherent in purifying the latter protein. The development of systems for overexpression of islet (this work) and liver (this work and [22]) glucokinases now provides a convenient and abundant source of the two proteins for the purposes of purification and careful evaluation of their kinetic and regulatory properties.

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